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Articles

Convergent Biocatalytic Mediated Synthesis of siRNA

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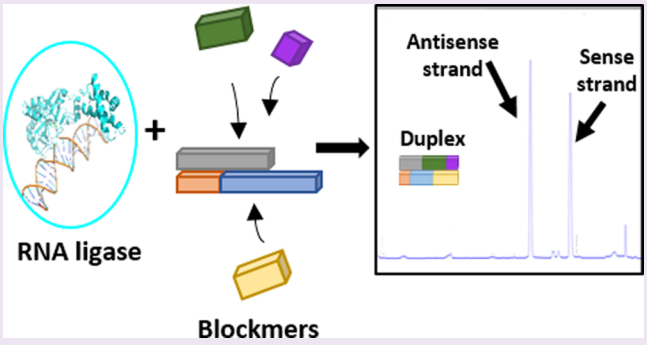
ABSTRACT: New technologies are required to combat the challenges faced with manufacturing commercial quantities of oligonucleotide drug substances which are required for treating large patient populations. Herein we report a convergent biocatalytic synthesis strategy for an Alnylam model siRNA. The siRNA chemical structure includes several of the unnatural modifications and conjugations typical of siRNA drug substances. Using Almac's 3−2−3−2 hybrid RNA ligase enzyme strategy that sequentially ligates short oligonucleotide fragments (blockmers), the target siRNA was produced to high purity at 1 mM concentration. Additional strategies were investigated including the use of polynucleotide kinase phosphorylation and the use of crude blockmer starting materials without chromatographic purification. These findings highlight a path toward a convergent synthesis of siRNAs for large-scale manufacture marrying both enzymatic liquid and classical solid-phase synthesis.



Oligonucleotide therapeutics are a class of drug based on the structure of DNA and/or RNA that act at the interface between genes and proteins.[1,2] In recent years, there has been significant advancement in next-generation therapeutics for a multitude of diseases through the development of natural and modified oligonucleotides (oligos). Many of these therapeutics are effective for the treatment of a cornucopia of rare diseases as augmented by the increasing number of oligos receiving approval from the FDA.[3] Due to the successes within the rare disease area, the development of oligo based therapeutics for larger disease populations is well underway and highlighted with the recent approval of Leqvio (inclisiran) for the treatment of adults with primary hypercholesterolaemia or mixed dyslipidaemia.[4] It is therefore anticipated that there will be an increase in market demand for production of oligo-based drugs requiring large scale manufacture. Currently, the preferred method for synthesis and manufacture of oligo-based drug substances is solid-phase oligo synthesis. Solid-phase synthesis is a well-established platform for manufacture of milligrams to kilogram quantities.[5] However, the solid-phase synthesis, being a linear process, can have limitations around scalability and purity for longer oligos. Capacity constraints on assets is also a supply chain concern for securing access to large quantities of 100s of kilograms or metric ton volumes.

Liquid-phase oligo synthesis studies and development are underway to address some of the limitations of solid-phase synthesis scale-up as reviewed by Molina and Sanghvi.[6] Liquid-phase oligo synthesis developments has investigated a number of different soluble polymeric supports and scaffolds.[7−9] These strategies were used for short sequences and to produce small gram quantities. Zhou et al. have reported a convergent liquid-phase synthesis of an 18-mer antisense oligonucleotide using tetramer and pentamer fragments (blockmers), without the need for column chromatography but with a similar impurity profile to solid-phase synthesis.[10] However, various impurities were encountered arising from undesirable coupling reactions and non-equimolar ligation requirements. More recently a novel organic solvent nanofiltration (OSN) was showcased by Kim et al.,[11] facilitating the separation of the oligonucleotide from reagents and byproducts during synthesis.

The assembly of short oligonucleotide fragments and subsequent enzymatic ligation to form double-stranded oligonucleotides of varying lengths (including single-strand hairpins and rings) is well documented.[14−19] Furthermore, the double-stranded RNA ligase (RNAL) enzymes have been shown to accommodate unnatural oligonucleotides at the ligation site,[20−22] a prerequisite for their utilization in the synthesis of oligonucleotide therapeutics. Within our group we have developed the Almac 3−2−3−2 hybrid technology approach to oligo synthesis,[12,13] consisting of a series of

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sequential ligations steps, each starting with 3 blockmers and ending with 2 partially complementary oligo strands (3−2−3−2) with an overhang. The overhang serves as a template for the next annealing step in the alternate strand. Repeating these ligation steps, alternating the two oligo strands leads to the desired double-stranded siRNA (Figure 1). This one-pot

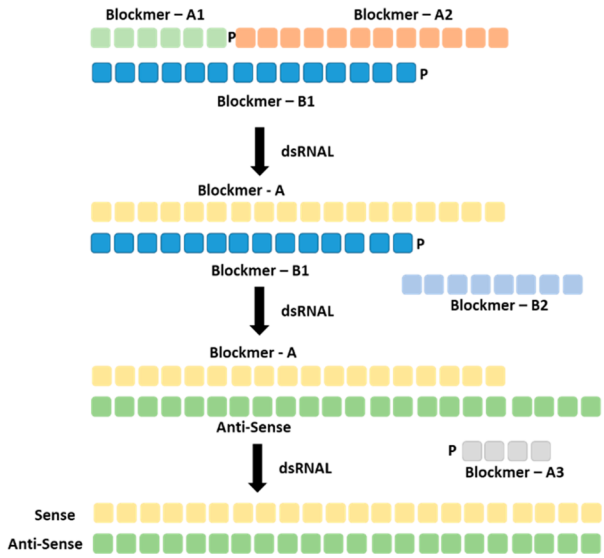

Figure 1. Almac's 3−2−3−2 hybrid RNA ligase oligo synthesis strategy.

approach utilizes Almac RNA ligase enzymes to provide a Biocatalytic OligOnucleotide Synthesis Technology (BOOST) platform that complements existing oligo synthesis technologies. The BOOST technology utilizes engineered RNA ligase enzymes (specifically double-stranded RNA ligases) and is supported by polynucleotide kinase (PNK) enzymes that can provide 5′ phosphorylation of chemically synthesized blockmers.

The BOOST technology encompasses panels of diverse engineered ligases and PNKs that allow selection of the optimum enzyme for specific retrosynthetic disconnection of the selected oligo into its represented blockmers. The short blockmers are chemically synthesized, annealed as part of the 3−2−3−2 approach followed by enzymatic ligation to produce the double-stranded product of interest. The 3−2−3−2 approach allows quality control points to ensure that complete ligation is achieved prior to addition of the next blockmer to the reaction sequence. At no point are more than 3 blockmers available in the reaction sequence.

METHODS

RNAL Screening Reaction. Prior to the ligation reactions, blockmers were annealed at 0.2 mM (×2 final concentration of each blockmer) in the presence of reaction buffer (50 mM Tris-HCl, pH 7.5, 100 mM KCl, and 10 mM $MgCl_2$, prepared in nuclease free water) for 15 min at 65 °C. Annealed blockmers were cooled to RT before being kept on ice until reactions were set up.

RNA ligase cell free extract (RNAL CFE) was resuspended in reaction buffer containing 1 mM dithiothreitol (DTT) and transferred to 96 well plates. Reactions were set up as detailed in Table S1, incubated at 25 °C at 400 rpm and samples were taken at T = 1 and 20 h time points. Reactions were quenched by addition of 1 volume (vol.) methanol (MeOH), shaken, and centrifuged to pellet any precipitation. Supernatants were analyzed by UPLC, method detailed in Table S3.

PNK Screening Reaction. For the initial screening, polynucleotide kinase cell free extract (PNK CFE) was resuspended in reaction buffer in 96 well plates. Reactions were set up using 5′-hydroxylated 1.2 and 1.3 blockmers (1.2-OH and 1.3-OH) at 0.5 mM final concentration in reaction mix detailed in Table S2. Control reactions were set up with empty vector control (EVC) and no enzyme present. Reactions were incubated at 25 °C at 400 rpm with samples taken at various time points and quenched by addition of 100% v/v methanol (MeOH), shaken and centrifuged to pellet any precipitation, and supernatants were analyzed by UPLC following the methods detailed in Table S3. Samples containing 0.5 mM blockmers were diluted 10-fold prior to UPLC analysis.

Prior to reactions being used for ligations, the samples were heat treated at 75 °C for 10 min (initial reactions) or 30 min (crude reactions), then centrifuged to pellet any precipitated material and supernatant used for ligations.

For confirmation of reaction, products/byproducts of ligation reaction were analyzed by mass spectrometry on an Agilent 6230 TOF operating in negative ionization mode, with a scan range of 400−2000 m/z coupled to an Agilent 1290 UPLC. The UPLC method detailed in Table S3 was used for chromatographic separation with modification of mobile phase A to exclude the EDTA, due to ion suppression.

RESULTS

Ligation Screening with Double-Stranded Ligases. The BOOST technology was used to synthesize an Alnylam model siRNA as detailed in Figure 2. The target sequence was

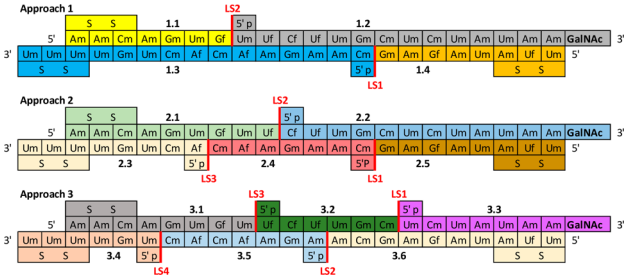

Figure 2. Approaches for formation of siRNA using BOOST ligase enzymes. The ligation sites (LS) for each approach are indicated.

analyzed to select appropriate disconnection and ligation sites, with three approaches selected, investigating various ligation points and blockmer lengths. Each approach was screened at the individual ligation sites comprising the two blockmers to be ligated with the complementary splinting blockmer. This screening strategy allowed for identification of potential and problematic by-products which may form as part of the ligation step. Screening of different blockmers and resultant self-annealing with a panel of engineered RNA ligases allows for the selection of the best combination of ligase and blockmers for optimum productivity and quality output.

Initial screening was conducted at 0.1 mM concentration of the chemically synthesized blockmers using 1 mg mL^{-1} RNAL CFE, with sampling at 1 and 16 h. Table 1 provides a summary of the initial screening results, with observed by-products at each site.

Based on the observed by-products from initial screening, directional synthesis strategies were trialed, resulting in successful synthesis of the full-length duplex with all three approaches. These findings illustrate the successful ligation with complementary portions as short as 3 bp and the accommodation of phosphorothioate linkages and GalNAc modifications at the oligonucleotide terminus. Figure 3

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Table 1. Summary Screening Results from Ligation of Blockmers at Individual Ligation Sites[a]

	Ligation site	Blockmers ligated	Splint	Top RNAL hits	Observed by-products
Approach 1	1	1.3 and 1.4	1.2	405, 419, 420	None
	2	1.1 and 1.2	1.3	405, 419, 420	1.2_1.3 hairpin
Approach 2	1	2.4 and 2.5	2.2	405, 408, 424	None
	2	2.1 and 2.2	2.4	405, 418, 419	2.4 [-3′ CmP], 2.4 [-3′ CmP]_2.4
	3	2.3 and 2.4	2.1	405, 406, 408	2.3_2.4[+AMP], 2.1_2.3 hairpin
Approach 3[b]	1	3.2 and 3.3	3.6	404, 405, 406	3.6_3.2_3.2
	2	3.5 and 3.6	3.2	414, 423, 471	3.2_3.2, 3.2_3.2_3.2, 3.5[-3′*Cm*]_3.6
	3	3.1 and 3.2	3.5	404, 405, 406	3.1[-3′ Um], 3.1_3.2_3.5, 3.1_3.2_3.5_3.5[-3′ *Cm*]
	4	3.4 and 3.5	3.1	405, 406, 424	3.4_3.5[-3′ *Cm*], 3.1[-3′ Um]_3.5_3.4

[a]An underscore designates a ligation product between blockmers. Structure for observed by-products are detailed in Supporting Information as deduced from MS analysis. [b]Approach 3 LS2 top hit reported as RNAL producing all reaction product peaks.

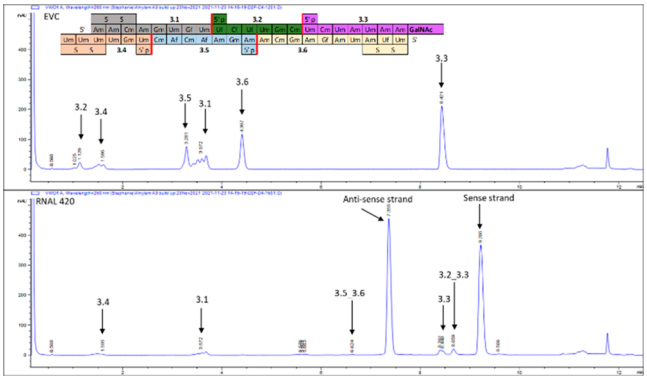

Figure 3. Formation of siRNA duplex using Approach 3 blockmers and RNAL 420. Blockmers were ligated starting with ligation of 3.2 and 3.3 with 3.6 splint, then sequential ligation in order 3.5, 3.1, and finally 3.4. Top—parallel no-enzyme control, bottom—final RNAL reaction product.

Figure 4. Screening results for PNK phosphorylation of blockmers (A) 1.2-OH (lilac bars) to 1.2-P (purple bars) and (B) 1.3-OH (yellow bars) to 1.3-P (green bars) with 1 mg mL^{-1} PNK CFE. The numbers on the *X*-axis represent distinct PNK enzymes that were screened.

illustrates the final reaction product following the sequential formation of the target siRNA with the 6 blockmer strategy (Approach 3) at 0.1 mM concentration using RNAL-420 outlined in Figure 2. The reaction temperature and time were altered at LS2 (3.6, 3.2_3.3−3.5 addition) and LS4 (final ligation of 3.4 to 3.6_3.5) to avoid by-product formation. The results show clean formation of the sense and antisense strand, with none of the by-products observed from the individual ligation site screening. UPLC-MS was performed on the final product (Figure S10) and demonstrated rejection of blockmer starting material impurities, indicating a potential purity enrichment with the enzymatic ligation method.

Route Development for High Titer siRNA Enzymatic Synthesis. Approach 1 was selected for further development, focusing on improving reaction titer, utilizing crude blockmers, and investigating the use of polynucleotide kinase for enzymatic 5′ phosphorylation. Nonphosphorylated starting material in the crude blockmers would result in incomplete strand formation. Enzymatic phosphorylation screening of the requisite 1.2 and 1.3 (5′OH) for Approach 1 as an alternative to chemical phosphorylation was investigated using a 96-PNK panel (0.5 mM concentration with 1 mg mL^{-1} PNK CFE) and revealed a number of PNKs capable of performing the desired phosphorylation (Figure 4). Reaction parameter investigation allowed for final phosphorylation at 5 mM blockmer concentration, with a one-pot phosphorylation of 1.2 and 1.3 (5′OH) with PNK-101. The reaction product was successfully telescoped into a ligation reaction with 1.4 to form the antisense strand followed by addition and ligation of 1.1 to yield the full-length duplex (Figure S5).

While initial screening was performed using blockmers purified by anion exchange chromatography, the crude synthetic mixtures desalted by diafiltration were subsequently used in an effort to eliminate chromatographic purification. Reactions using crude blockmers for Approach 1 without chromatographic purification resulted in successful ligation to form the target siRNA at 1 mM concentration using 3−2−3−2 directional synthesis strategy (Figure S6).

Formation of the duplex with crude blockmers for Approaches 2 and 3 was not investigated, but it is expected that, combined with the higher purity that can be achieved with solid-phase synthesis of shorter blockmers, further purity enrichment can be achieved as the product is built up through sequential 3−2−3−2 ligation reactions.

For scale-up of RNAL ligation reactions various parameters were investigated including ATP concentration, reaction temperature, substrate, and enzyme loading. Final reaction development achieved synthesis of the target siRNA at 1 mM concentration using either crude or purified blockmers combined with PNK phosphorylation of 1.2 and 1.3 (5′ OH) in jacketed 1 L batch vessels. Ligation with starting blockmer concentrations as high as 10 mM was observed; however, reactions were slow and failed to go completion, indicating a target for further enzyme or reaction engineering.

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CONCLUSION

In conclusion, the target siRNA was successfully synthesized utilizing Almac's 3−2−3−2 hybrid BOOST technology. The target is fully comprised of unnatural nucleotides, with multiple phosphorothioate linkages and a GalNAc conjugate. Almac ligase and PNK enzyme panels were screened against the different ligation sites for each of the different approaches. Approach 3 which consisted of 6 blockmers ligated together showed clean formation of both the sense and antisense strand with purity enrichment through selective ligation. The biocatalytic approach was also tested on blockmers that were not chromatographically purified showing formation of siRNA duplex and purity enrichment which could be further achieved by combining shorter blockmers that can be synthesized in high yield and purity.

An increasing demand for oligonucleotides as therapeutics has focused attention on methods for their synthesis. The work presented in this communication highlights how RNAL and PNK enzymes developed at Almac can be used for oligonucleotide synthesis via a biocatalytic route. The hybrid approach utilizes both synthetic and biocatalytic methods and offers an attractive technology to address some of the limitations encountered with scale-up to enable delivery of siRNA therapeutics to wider patient populations.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.3c00071.

Experimental details including RNAL ligation and PNK phosphorylation screening reactions for each ligation site and full duplex formation are detailed as well as the proposed structure of potential byproducts observed in individual ligation site reactions (PDF)

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Author Contributions

#The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. S.P. and D.G. contributed equally.

Notes

The authors declare no competing financial interest.

ABBREVIATIONS

FDA, US Food and Drug Administration; OSN, organic solvent nanofiltration; RNAL, RNA ligase; BOOST, Biocatalytic OligOnucleotide Synthesis Technology; PNK, Polynucleotide Kinase; CFE, Cell free extract; LS, ligation site; GalNAc, N-Acetylgalactosamine; ATP, Adenosine 3′ triphosphate

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